BRIEF REPORT

Inheritance of a Cancer-Associated *MLH1* Germ-Line Epimutation

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SUMMARY

Persons who have hypermethylation of one allele of *MLH1* in somatic cells throughout the body (a germ-line epimutation) have a predisposition for the development of cancer in a pattern typical of hereditary nonpolyposis colorectal cancer. By studying the families of two such persons, we found evidence that the epimutation was transmitted from a mother to her son but was erased in his spermatozoa. The affected maternal allele was inherited by three other siblings from these two families, but in those offspring the allele had reverted to the normal active state. These findings demonstrate a novel pattern of inheritance of cancer susceptibility and are consistent with transgenerational epigenetic inheritance.

Hereditary nonpolyposis colorectal cancer results from germline sequence mutations in mismatch-repair genes, particularly *MLH1* and *MSH2*. Somatic inactivation of the remaining normal allele by genetic or epigenetic events leads to the development of microsatellite instability in tumors of the colorectum or endometrium at a young age.[1] Methylation of cytosines that precede a guanosine in the DNA sequence (the CpG dinucleotide) is a key epigenetic modification, and hypermethylation of gene promoters is associated with transcriptional silencing.[2] In sporadic colorectal cancer, methylation of both alleles of the promoter of *MLH1* occurs as a somatic event early in tumorigenesis and is seen in approximately 15% of patients.[3]

Studies have shown that hypermethylation of *MLH1* is not limited to neoplastic cells. Rather, in some persons, hypermethylation of a single allele of *MLH1* originates in the germ line and is thus widespread in normal somatic cells.[4-7] This phenomenon of germ-line epimutation silences the affected allele in the absence of intragenic sequence mutations. Persons with a germ-line epimutation, like those with hereditary nonpolyposis colorectal cancer, have only one functional allele of the *MLH1* gene from conception, and cancers typical of the hereditary nonpolyposis colorectal cancer syndrome have developed in all such cases described to date.[4-7] Colorectal and other tumors in persons with *MLH1* germ-line epimutations do not express the MLH1 protein and have microsatellite instability (the hallmark of failed mismatch-repair function), accompanied in some cases by somatic loss of the wild-type allele.[4-7]

Although germ-line sequence mutations are faithfully transmitted from one generation to the next in a mendelian pattern, epimutations do not involve changes in the DNA sequence and are relatively unstable, perhaps as a result of epigenetic reprogramming, in primordial germ cells and gametes,[8] in the male genome in the zygote,[9,10]

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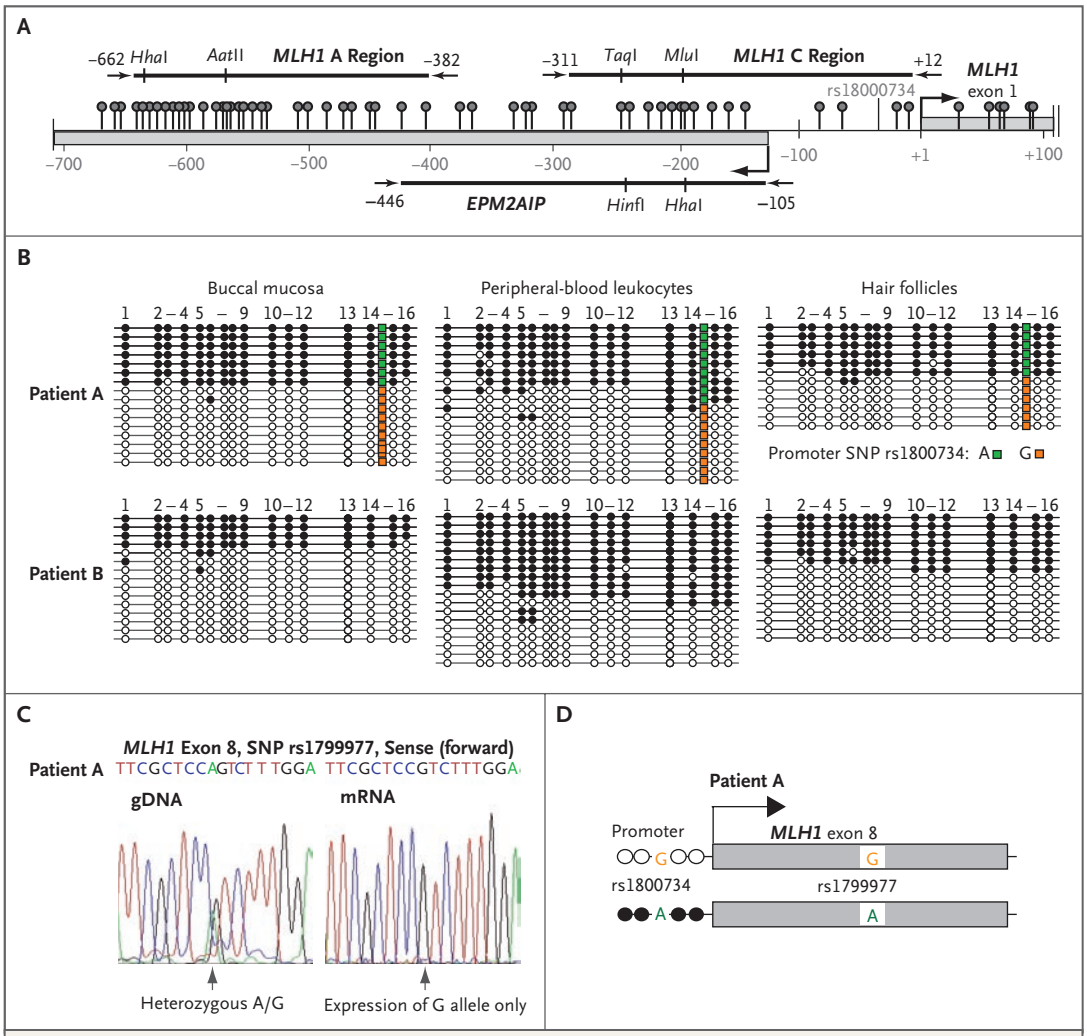


Figure 1. Germ-Line Epimutation and Allelic Loss of Expression of *MLH1* in Patients A and B.

Panel A shows a map of the CpG island encompassing the *MLH1* and *EPM2AIP* promoters. The transcriptional start site is indicated by +1. Individual CpG doublets are depicted as circles. Horizontal bars show the regions amplified for combined bisulfite restriction analysis and bisulfite allelic sequencing. Primer positions are indicated by short arrows. Sites for restriction enzymes that exclusively cleave combined bisulfite restriction analysis amplicons from methylated templates are shown. Panel B shows bisulfite allelic sequencing of the C region of the *MLH1* promoter in somatic tissues from Patient A and Patient B. Each horizontal line represents a single allele, and circles depict individual CpG dinucleotides as numbered, with black circles indicating methylated dinucleotides and white circles indicating unmethylated dinucleotides. Single-nucleotide polymorphism (SNP) rs1800734 is indicated with G in orange and A in green. Panel C shows representative sequence-based fluorescent electropherograms showing the SNP within exon 8 of *MLH1* in the genomic DNA (gDNA) and messenger RNA (mRNA) from peripheral-blood leukocytes of Patient A; gDNA shows heterozygosity (arrow), whereas transcription is restricted to the G allele. Panel D is a schematic diagram of the effect of the germ-line epimutation on allelic activity of *MLH1* in Patient A. Uniparental methylation of the promoter A allele (SNP rs1800734) causes allelic inactivation, so that only the allele with the G SNP at exon 8 (SNP rs1799977) is expressed (arrow).

and in the preimplantation embryo.[11] This process removes and resets epigenetic marks between generations. Nevertheless, nonmendelian patterns of transgenerational epigenetic inheritance have been reported in mice.[12-14] In the eight previously reported cases of germ-line *MLH1* epimutation in humans, no intergenerational transmission was found, although in one patient the epimutation was reported in a low proportion of spermatozoa.[4]

Since the presence of an *MLH1* epimutation in

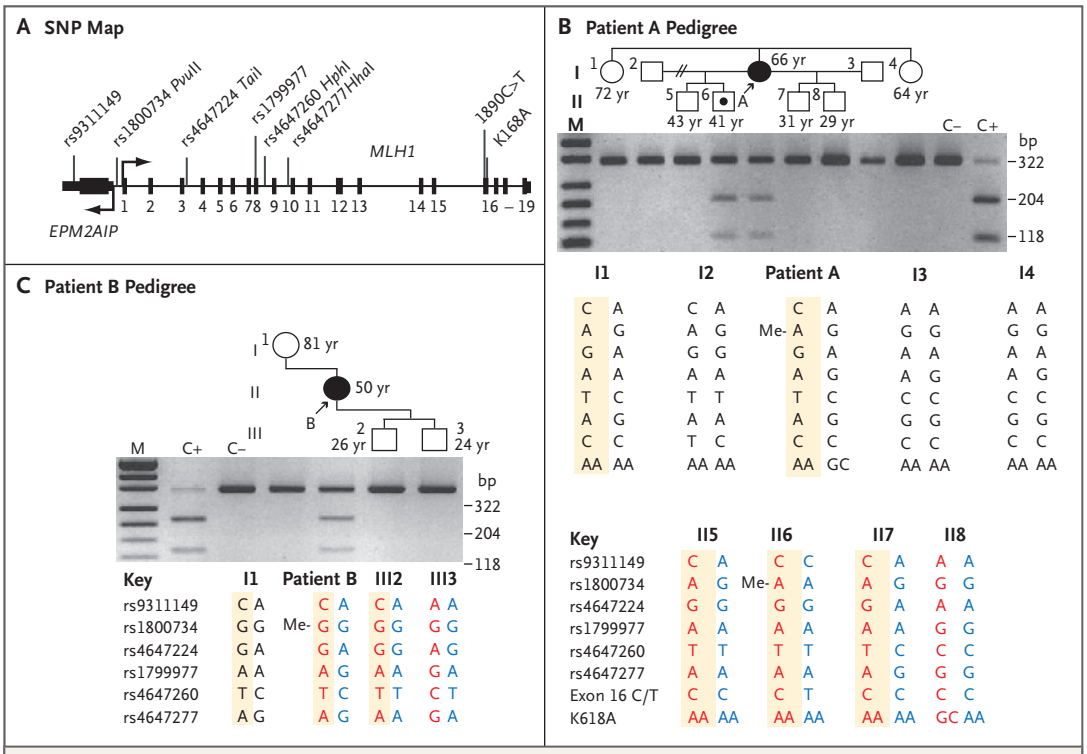


Figure 2. Pedigrees of Patients A and B Showing Intergenerational Transmission of the *MLH1* Epimutation and Haplotypes.

Panel A shows a map of SNPs within the *MLH1* and *EPM2AIP* genes, used to determine inheritance patterns of the epimutant alleles and to analyze allelic expression. Panels B and C show pedigrees for Patient A and Patient B, with large black circles denoting Patient A and Patient B and with the current age of each family member given. Generations are listed I to III, and patients are identified by number. In Panels B and C, combined bisulfite restriction analysis of the C region of *MLH1* (digested with *Mlu*I) is shown, with the lanes corresponding to data for family members shown in the pedigrees directly above. Combined bisulfite restriction analysis was performed on DNA extracted from peripheral-blood leukocytes from all patients except family member II7-A and family member II8-A, from whom only hair-follicle DNA was available. Lane M indicates the pUC19/MspI DNA ladder, lane C– indicates DNA extracted from peripheral-blood leukocytes from a control with an unmethylated *MLH1* promoter, and lane C+ indicates DNA extracted from the biallelically methylated RKO colorectal-carcinoma cell line (ATCC). Band sizes (in base pairs) are shown at the right. At the bottom of Panels B and C, haplotypes generated from informative SNPs are listed according to the key for each pedigree. Alleles associated with the epimutation are highlighted in yellow, and the presence of methylation (Me) is indicated. Maternally inherited alleles are shown in red, and paternally derived alleles are shown in blue; black letters indicate unknown parental origin. Although the haplotypes associated with the epimutation were inherited by several children, only family member II6-A retained the epimutation.

the germ line implies a potential for inheritance, we sought evidence for transmission between generations. In this study, we identified additional persons with germ-line *MLH1* epimutations and in one family showed maternal transmission of the epimutation to her son but its erasure in his spermatozoa. Three other siblings from the two families we studied also inherited the affected maternal alleles, but in these cases the epimutation had reverted to the normal state, with concomitant allelic reactivation. These findings are consistent with germ-line transmission of a silent epigenetic state that confers disease susceptibility in humans.

METHODS

PATIENTS AND FAMILY MEMBERS

Our study was approved by the ethics committee at St. Vincent's Hospital in Sydney. Tissues were obtained with written informed consent from probands and family members at St. Vincent's Hospital and Women's and Children's Hospital in Adelaide, Australia.

We selected 24 patients in whom colorectal or endometrial cancer had developed before the age of 50 years and who lacked deleterious germ-line sequence mutations in *MSH2* or *MLH1*. In each case, the tumors had microsatellite instability, with complete loss of MLH1 protein expression and retention of MSH2. Additional tissues were collected from patients shown to carry an *MLH1* germ-line epimutation and from their first-degree relatives. The "swim-up" procedure was used to isolate motile spermatozoa.[15] To remove contaminating somatic cells, spermatozoa that were used for DNA analyses were additionally sorted by flow cytometry (FACSVantage DiVa, Becton Dickinson).[16] DNA was extracted from spermatozoa, hair follicles, and buccal mucosa with the use of QuickExtract solution (Epicentre Biotechnologies) and from peripheral-blood leukocytes with the use of phenol–chloroform.

METHYLATION ANALYSES

Methylation of the promoter of *MLH1* (A and C regions) and of the *EPM2AIP* promoter on the opposite strand was identified with the use of combined bisulfite restriction analyses (Fig. 1A) and confirmed by allelic bisulfite sequencing.[7]

Quantitative real-time methylation-specific polymerase-chain-reaction (PCR) assays were performed on bisulfite-treated DNA with the use of primers specific to methylated templates for the C region of *MLH1* and the imprinted *SNRPN* gene.[17] Primers that amplify the control gene *MyoD* regardless of its methylation status were used to normalize for DNA input. Real-time methylation-specific PCR assays with iQ SYBR Green Supermix reagent were analyzed with the use of a real-time PCR system (MyiQ, BioRad). Absolute values for experimental samples were calculated from the PCR cycle number at which the fluorescence crossed the threshold with the use of a standard curve. The percentage of methylated alleles in the C region of *MLH1* and *SNRPN* was calculated against *MyoD* with reference to 100% in vitro methylated human DNA (Chemicon).[18] All primer sequences are available on request.

Figure 3 (facing page). Maternal Inheritance of the *MLH1* Germ-Line Epimutation by Family Member II6-A and the Erasure of the Epimutation in His Spermatozoa.

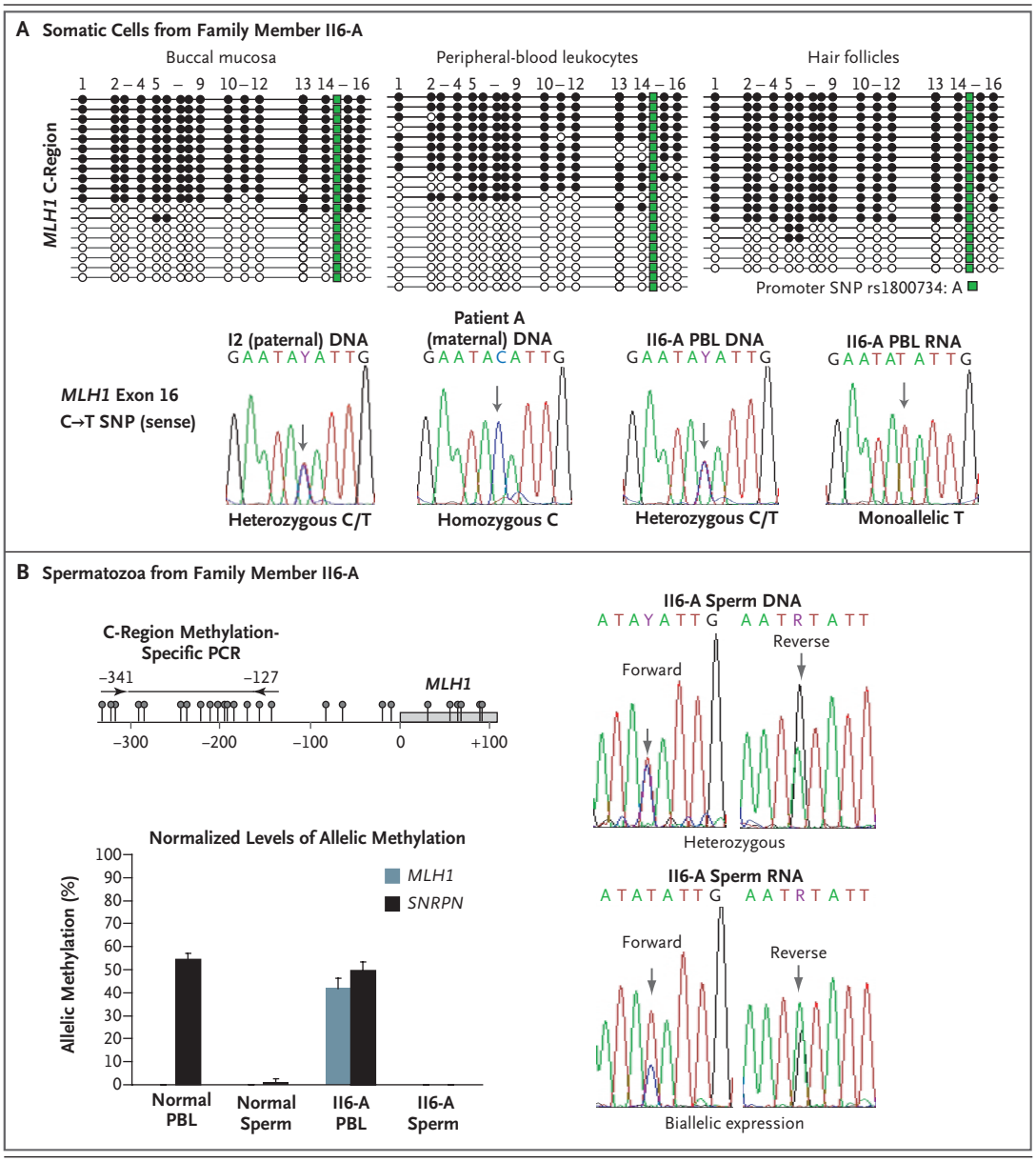
Panel A shows methylation and monoallelic expression of *MLH1* in the somatic tissues of a son of Patient A, family member II6-A. Bisulfite allelic sequencing of the C region of the *MLH1* promoter in somatic tissues from family member II6-A appears at the top of the panel. Each horizontal line represents a single allele, and circles depict individual CpG dinucleotides, with black circles indicating methylated dinucleotides and white circles indicating unmethylated dinucleotides. family member II6-A is homozygous for the rs1800734 SNP (indicated by green boxes). Sequence electropherograms show the exon 16 C→T SNP (arrow). The SNP is at nucleotide position 1890 of the *MLH1* messenger RNA sequence (GenBank accession number NM000249) and does not confer any amino acid change. Family member II6-A and his father are heterozygous for this SNP. Family member II6-A expresses *MLH1* from only the paternally derived (T) allele. Panel B shows erasure of the germ-line *MLH1* epimutation in spermatozoa obtained from family member II6-A. At the top of the panel, the map shows the region of the *MLH1* promoter amplified by real-time methylation-specific PCR. Below the map, a graph shows the percentage of alleles methylated at either *MLH1* or the differentially methylated region of the imprinted *SNRPN* gene, as determined by real-time methylation-specific PCR. *SNRPN* is an imprinted gene methylated specifically on the maternal allele; it is unmethylated in mature spermatozoa,[21] providing a control for somatic contamination of the spermatozoa samples. As expected, *SNRPN* shows approximately 50% methylation in somatic DNA and negligible methylation in sperm DNA. In family member II6-A, *MLH1* shows 42% methylation in peripheral-blood leukocytes (PBLs) but no methylation in spermatozoa. In the right portion of the panel, sequence electropherograms of the exon 16 C→T SNP from DNA and RNA in spermatozoa from family member II6-A show the presence of both *MLH1* alleles in the spermatozoa DNA and biallelic expression in the RNA. Spermatozoa for the RNA analysis were subjected to the "swim-up" technique only and may contain a proportion of somatic cells.

HAPLOTYPING

Single-nucleotide polymorphism (SNP) typing was performed by PCR amplification of constitutional DNA followed by restriction digestion or direct sequencing of the purified amplicons. Markers of sequence-tagged sites were typed by PCR amplification with the use of fluorescent-labeled primers,[19] separated by capillary electrophoresis on an automated DNA sequencer, and sized with the use of LIZ markers (ABI 3700, Applied Biosystems).

ALLELIC EXPRESSION

For each patient or family member, heterozygous polymorphisms within exons of *MLH1* and *EPM2AIP* were used to identify the alleles being transcribed.[20] RNA was extracted from peripheral-blood leukocytes, lymphoblastoid cells, and



spermatozoa with the use of Trizol reagent (Invitrogen), treated with DNaseI, and converted to complementary DNA (cDNA). Allelic expression was determined by sequencing cDNA at I219V SNP within exon 8 (rs1799977) and at polymorphisms within exon 16 and *EPM2AIP*.

RESULTS

By studying the peripheral blood of 24 patients, we identified two unrelated women (Patient A and Patient B) who had the typical molecular and clinical characteristics of persons with germ-line *MLH1* epimutations — namely, multiple MLH1-negative cancers of the colorectum and endometrium and hemiallelic methylation of *MLH1* in all somatic cells. In both women, there was dense methylation of one allele of the *MLH1* and *EPM2AIP* promoters in somatic cells from the three embryonic germ layers (Fig. 1B, and Fig. 1A of the Supplementary Appendix, available with the full text of this article at www.nejm.org). Both women had metachronous carcinomas that had microsatellite instability and lacked MLH1 expression. (Patient A received a diagnosis of cancer of the endometrium at the age of 45 years, of the colon at 59 years,

and of the rectum at 60 years; Patient B received a diagnosis of cancer of the colon at 41 years and of the rectum at 45 years.) Patient A was heterozygous for a SNP (rs1800734) within the *MLH1* promoter, with methylation confined to the A allele. In both Patient A and Patient B, the methylated allele was transcriptionally silent, as evidenced by monoallelic expression of *MLH1* and *EPM2AIP* transcripts in their messenger RNA (mRNA) (Fig. 1C and 1D, and Fig. 1B of the Supplementary Appendix).

To identify *MLH1* epimutations within families of the probands, combined bisulfite restriction analysis was performed on constitutional DNA from nine first-degree relatives, none of whom had a history of cancer (Fig. 2B and 2C). Partial methylation of *MLH1* was found in one of Patient A's four sons (family member II6-A) (Fig. 2C). Methylation of the A allele (SNP rs1800734) on approximately 50% of chromosomes was confirmed by bisulfite sequencing (Fig. 3A). We identified an expressible C→T SNP within *MLH1* exon 16 in family member II6-A, which was used to demonstrate that he was transcribing RNA only from the *MLH1* allele inherited from his father (Fig. 3A). These data are consistent with transmission of the *MLH1* epimutation from Patient A to her son.

To ascertain the possibility of transmission of the *MLH1* epimutation from family member II6-A to his offspring, we studied the level of allelic methylation in his pure motile spermatozoa with the use of a sensitive quantitative real-time methylation-specific PCR assay within the C region of *MLH1* (Fig. 1A). In DNA from peripheral-blood leukocytes obtained from family member II6-A, approximately half of the *MLH1* alleles (mean ±SD, 42.0±4.6%) were methylated. In contrast, his sperm had no trace of *MLH1* methylation, despite containing equal proportions of alleles derived from his mother and father (Fig. 3B). Furthermore, analysis of the RNA in his sperm at the *MLH1* exon 16 C→T SNP showed reactivation of the maternally derived *MLH1* allele (Fig. 3B). These results indicate reversion of the *MLH1* epimutation to normality during spermatogenesis, suggesting a negligible risk of transmission from family member II6-A (Fig. 4).

To investigate allelic inheritance patterns and the possibility that the epimutations were caused by alterations in the DNA strand carrying the allele that could influence methylation of the allele (*in cis* defects), we used SNPs within *MLH1*, *EPM2AIP*, and flanking sequence-tagged site markers to construct a haplotype map of a region of approximately 8 Mb around *MLH1* for all members of both families (Fig. 2B and 2C, and Fig. 2 of the Supplementary Appendix). Patient A, her sister (family member I1-A), and three of her four sons (family members II5-A, II6-A, and II7-A) all shared a haplotype in this region, even though *MLH1* was methylated only in Patient A and her son (family member II6-A). In the case of Patient B, haplotype and expression analysis (Fig. 2B, and Fig. 1 of the Supplementary Appendix) confirmed that the *MLH1* epimutation resided on her maternally derived allele. This is likely to have arisen spontaneously, since we found no evidence of methylation in her mother (Fig. 2B). Notably, the haplotype in Patient B differed from the one on which the epimutation occurred in Patient A's family. Patient B transmitted the haplotype to one of her two sons (family member III2-B), but there was no evidence of *MLH1* methylation in his case (Fig. 2B). For both family member II5-A and family member III2-B, absence of methylation from their respective maternal alleles correlated with biallelic expression from the *MLH1* locus (Fig. 3 of the Supplementary Appendix), indicating reversion of the epimutation in these family members or in their mothers' germ cells. No recombinations were observed in the vicinity of *MLH1*, indicating that the affected alleles were identical in family members bearing the epimutation and in those in whom it had reverted (Fig. 2 of the Supplementary Appendix).

Epimutations are meiotically reversible and often show somatic mosaicism. We therefore considered whether some family members had low levels of allelic *MLH1* methylation, suggesting incomplete somatic erasure of an epimutation, or a susceptibility of the allele to subsequent somatic methylation. With the use of real-time methylation-specific PCR, we found no evidence of allelic mosaicism for *MLH1* methylation in any patient in our study, except those with epimutations (38±9% for Patient B, 42±7% for Patient A, and 42±4.6% for family member II6-A).

DISCUSSION

We have found evidence of germ-line epimutation of *MLH1* in a woman with cancer and in her son (Fig. 4), which supports the concept of trans-

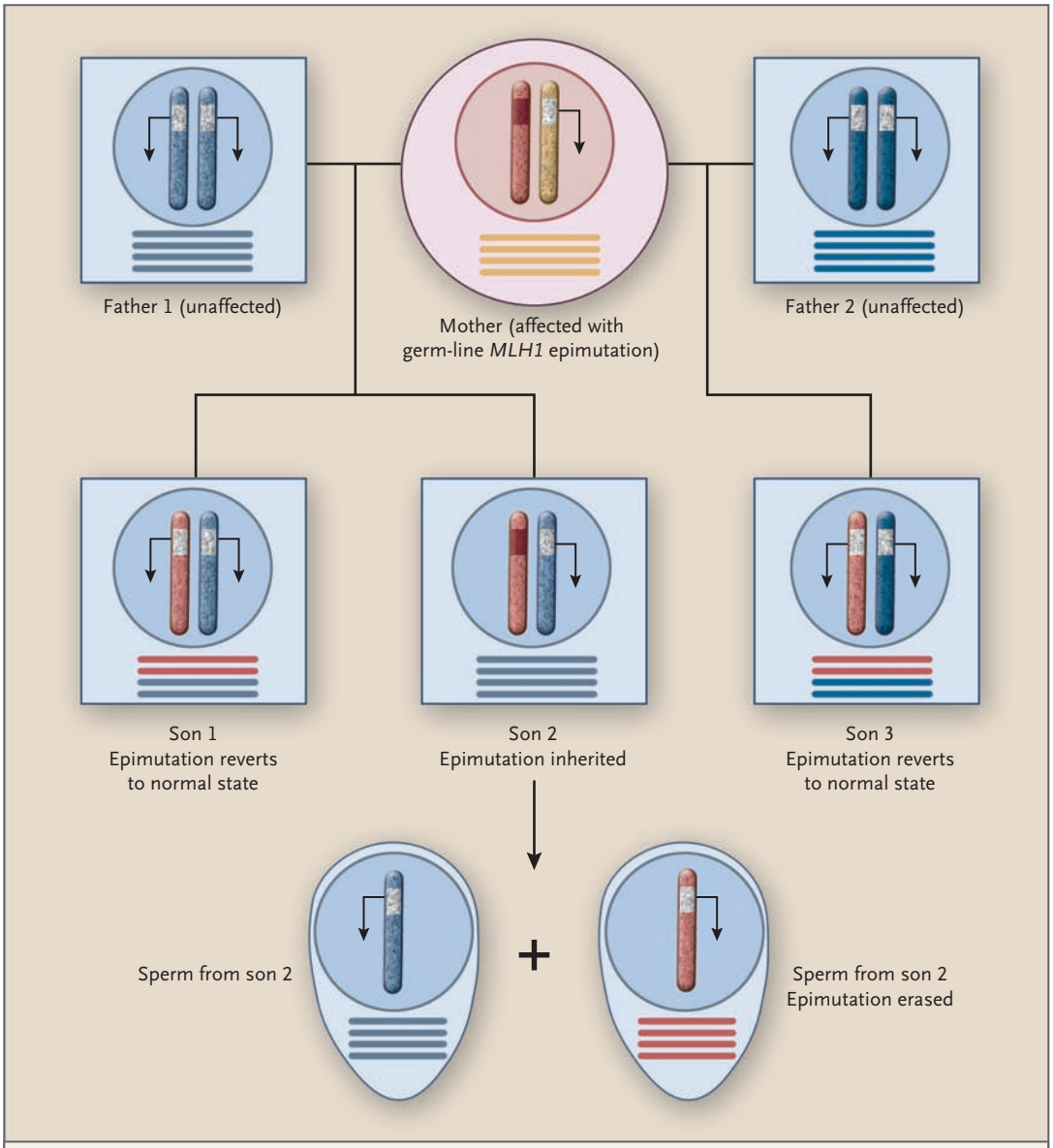


Figure 4. Schematic Representation of Inheritance of *MLH1* Alleles in Three Sons of Patient A.

The diagram depicts *MLH1* alleles within the nucleus of somatic cells and sperm, with pink representing maternal origin and blue representing paternal origin. Active transcription is indicated by an arrow, and the RNA transcripts from the alleles are shown within cytoplasm. In the mother, one allele was methylated in the promoter (red band) and therefore not transcribed; only RNA from her yellow allele was expressed. In her second son (family member II6-A), the maternally inherited allele (pink, red band) was silent, and the paternal allele (pale blue) was expressed. Haploid DNA within spermatozoa from the son contained alleles from both the father and mother, and both were transcribed. There was no evidence of methylation of either allele. The first and third sons (family members II5-A and II7-A) also inherited the affected maternal allele, but in these offspring methylation had been erased and biallelic expression of *MLH1* was found, indicating that the epimutation was reversed to the normal state. This model shows transgenerational inheritance of an epigenetically mutated tumor-suppressor gene and subsequent reversal of the epimutation within spermatozoa.

generational epigenetic inheritance. The *MLH1* epimutation that predisposed the mother to multiple tumors with microsatellite instability has increased the risk of cancer in her son (family member II6-A).

The findings from the two families in this study, as well as previous studies, offer insights into the pattern of inheritance of germ-line epimutations. With reference to the parent of origin, the mother of family member II6-A, like the mothers of two patients with *MLH1* epimutations reported previously, had cancer in a pattern typical of hereditary nonpolyposis colorectal cancer.[6] Furthermore, in two other cases in which germline epimutations were shown to arise spontaneously (Patient B and a patient whose case was reported previously[7]), the methylated allele was maternally derived. Taken together, these data raise the possibility that epigenetic errors may arise more frequently during oogenesis or are more likely to be maintained during this process. Our finding of erasure of the epimutation during spermatogenesis in family member II6-A is consistent with this hypothesis. However, paternal inheritance cannot be excluded, given our previous finding of low-level *MLH1* methylation in sperm of an affected person.[6]

Another characteristic of the inheritance pattern in the families in this study is that four sons inherited their mother's *MLH1* haplotype, yet in three of the sons, the maternally derived allele had undergone demethylation and transcriptional reactivation. It appears that the normal process of gametogenesis allowed correction of the *MLH1* epimutation, perhaps contemporaneously with erasure of parent-specific methylation of imprinted genes in primordial germ cells.[11] If so, then transmission of an epimutation to family member II6-A must reflect resistance to reprogramming, either through incomplete erasure or by retention of an epigenetic memory. Although at present we have limited information, the overall cancer risk for families with germ-line *MLH1* epimutations appears to be lower than for those with germ-line sequence mutations.

An alternative explanation for our findings is that epimutations are not inherited per se. Rather, they are erased in gametogenesis but reestablished in successive generations because of *cis*-acting or even *trans*-acting genetic factors that increase susceptibility to *MLH1* epimutations.[21,22] Examples of epigenetic silencing that are driven by genetic events *in cis* include deletion of imprint-control centers in imprinted disorders[23] and expansion of triplet repeats within the *FMR1* promoter in the fragile X syndrome.[24] Such a mechanism may also explain the recently reported strongly heritable pattern of epimutation in *MSH2*,[25] since the methylation state segregated faithfully with the genetic haplotype. In contrast, in the two families described in our study, we found no evidence of a fully penetrant *in cis* defect. Rather, they showed epimutations that were meiotically reversible and transmitted in a nonmendelian fashion. A simple explanation for this pattern is that epimutations can occur on any haplotype, and although they usually are cleared in the germ line, they may be retained at low but uncertain frequency.

Regardless of uncertainties about patterns, frequencies, and mechanisms of inheritance, offspring of patients with *MLH1* epimutations must be regarded as being at risk for cancer until proven otherwise. The broader implication of this study is that disease states in humans may be the consequence of nonmendelian inheritance of epigenetic changes in one or more genes.

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